ARTICLES

Secreted amyloid β-protein similar to that in the senile plaques of Alzheimer's disease is increased *in vivo* by the presenilin 1 and 2 and *APP* mutations linked to familial Alzheimer's disease

D. SCHEUNER[1], C. ECKMAN[1,2], M. JENSEN[3], X. SONG[4], M. CITRON[5], N. SUZUKI[6], T.D. BIRD[7,12], J. HARDY[14], M. HUTTON[14], W. KUKULL[8], E. LARSON[9], E. LEVY-LAHAD[9,13], M. VIITANEN[3], E. PESKIND[10,13], P. POORKAJ[7,13], G. SCHELLENBERG[7,9,11,13], R. TANZI[15], W. WASCO[15], L. LANNFELT[3], D. SELKOE[5] & S. YOUNKIN[2]

[1]*Department of Neuroscience, Case Western Reserve University, Cleveland, Ohio 44106, USA*
[2]*Mayo Clinic Jacksonville, 4500 San Pablo Road, Jacksonville, Florida 32224, USA*
[3]*Karolinska Institute, Department of Clinical Neuroscience and Family Medicine, Huddinge University Hospital, Novum KFC, S-141 86 Huddinge, Sweden*
[4]*Department of Pathology, Case Western Reserve University, Cleveland, Ohio 44106, USA*
[5]*Center for Neurologic Diseases, Harvard Medical School and Brigham and Women's Hospital, Boston, Massachusetts 02115, USA*
[6]*Discovery Research Division, Takeda Chemical Industries, Ltd., Wadai 10, 300-42 Tsukuba, Ibaraki, Japan*
[7]*Department of Neurology,* [8]*Department of Epidemiology,* [9]*Department of Medicine,* [10]*Department of Psychiatry and Behavioral Sciences &* [11]*Department of Pharmacology, University of Washington, Seattle, Washington 98185, USA*
[12]*Neurology Service and* [13]*Geriatric Research, Education, and Clinical Center, Veterans Affairs Puget Sound Health Care System, Seattle, Washington 98108, USA*
[14]*Suncoast Alzheimer's Disease Laboratories, Department of Psychiatry, University of South Florida, Tampa, Florida 33613, USA*
[15]*Genetics and Aging Unit, Massachusetts General Hospital, Department of Neurology, Harvard Medical School, Charlestown, Massachusetts 02129, USA*
D. Scheuner present address: Howard Hughes Medical Institute, University of Michigan, Ann Arbor, Michigan 48109, USA
D. Scheuner, C.E., M.J. and X.S. contributed equally to this study.
Correspondence should be addressed to S.Y.

To determine whether the presenilin 1 (*PS1*), presenilin 2 (*PS2*) and amyloid β-protein precursor (*APP*) mutations linked to familial Alzheimer's disease (FAD) increase the extracellular concentration of amyloid β-protein (Aβ) ending at Aβ42(43) *in vivo*, we performed a blinded comparison of plasma Aβ levels in carriers of these mutations and controls. Aβ1–42(43) was elevated in plasma from subjects with FAD-linked *PS1* ($P < 0.0001$), $PS2_{N141I}$ ($P = 0.009$), $APP_{K670N,M671L}$ ($P < 0.0001$), and APP_{V717I} (one subject) mutations. Aβ ending at Aβ42(43) was also significantly elevated in fibroblast media from subjects with *PS1* ($P < 0.0001$) or *PS2* ($P = 0.03$) mutations. These findings indicate that the FAD-linked mutations may all cause Alzheimer's disease by increasing the extracellular concentration of Aβ42(43), thereby fostering cerebral deposition of this highly amyloidogenic peptide.

Amyloid β-protein (Aβ) ending at Aβ42(43) is deposited early[1] and selectively[2–4] in the senile plaques that are an invariant feature of all forms of Alzheimer's disease (AD). It is now well established that Aβ is a secreted peptide that is normally released from the amyloid β-protein precursor (βAPP) through cleavage by proteases referred to as β and γ secretase[5,6]. Most secreted Aβ in human cerebrospinal fluid and in medium conditioned by cultured cells is Aβ1–40, but a small component is Aβ1–42(43)[9,10], which forms insoluble aggregates much faster than Aβ1–40 *in vitro*[11–14].

Because Aβ deposition is an early and constant feature of AD, it has been hypothesized that the *APP* and presinilin *PS1* and *PS2* mutations that are known to cause early-onset familial Alzheimer's disease (FAD)[15,45] act to foster Aβ deposition either by increasing the extracellular concentration of Aβ or through some other mechanism. We previously analyzed fibroblasts[29] from subjects carrying FAD-linked *APP* mutations or cells transfected with mutant APP cDNAs (ref. 27–29). These studies showed that the *APP* mutation just amino terminal to Aβ ($APP_{K670N,M671L}$) coordinately increases the extracellular concentration of Aβ1–40 and Aβ1–42(43)[26–28] and that the FAD-linked mutations carboxy terminal to Aβ ($APP_{V717I,V717G}$) selectively increase the concentration of Aβ1–42(43)[29,30]. In the present study, we analyzed the effect of the *APP*, *PS1*, and *PS2* mutations on the concentration of Aβ1–40 and Aβ1–42(43) in plasma and in medium conditioned by skin fibroblasts, reasoning that mutations in these widely expressed

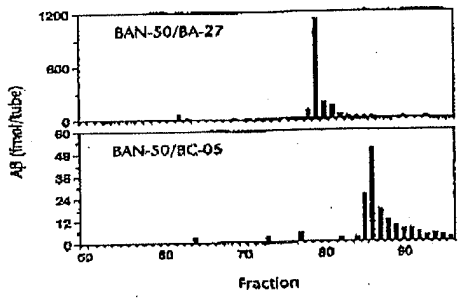

Fig. 1 Analysis by reversed-phase HPLC of the Aβ in human plasma. Plasma (50 ml) was applied to a BAN-50 immunoaffinity column. Adsorbed materials were eluted with 1 ml of 60% CH_3CN containing 0.2% TFA, and the eluate was lyophilized and further fractionated by reversed-phase HPLC on a Vydac C4 column (4.6 × 250 mm). Aliquots from fractions were analyzed by BAN-50/BA-27 (*upper panel*) or BAN-50/BC-05 (*lower panel*) ELISA.

genes would likely have a generalized effect, operative in peripheral as well as brain cells.

Plasma measurement of Aβ1–40 and Aβ1–42(43)

To determine whether Aβ1–40 and Aβ1–42(43) could be detected in human plasma, we analyzed samples obtained conventionally from young volunteers by using EDTA as the anticoagulant. In all plasma samples, Aβ was readily detected using the well-characterized BAN-50/BA-27 and BAN-50/BC-05 sandwich ELISAs that specifically detect Aβ1–40 and Aβ1–42(43), respectively, in medium conditioned by transfected cells expressing βAPP (ref. 29). To be sure that the signals measured in plasma were due to the targeted Aβs and not to cross-reacting proteins, a BAN-50 (anti-Aβ1–16) column was used to capture the Aβ in 50 ml of plasma, the affinity-purified protein was separated by reversed-phase HPLC using a C4 column, and each of the relevant fractions was analyzed with BAN-50/BA-27 and BAN-50/BC-05 assays. As expected[29], the BAN-50/BA-27 assay only detected plasma Aβ eluting from the C4 column at the same time as synthetic Aβ1–40, and the BAN-50/BC-05 assay only detected Aβ eluting at the same time as synthetic Aβ1–42 (Fig. 1). Analysis of synthetic Aβ1–40 and Aβ1–42 peptides in this same paradigm showed that recovery from the BAN-50 column was approximately 40% for both Aβ1–40 and Aβ1–42, and that the recovery of Aβ1–40 and Aβ1–42 from the C4 column was 66% and 28%, respectively. Assuming similar recovery of the Aβ1–40 and Aβ1–42(43) in plasma, we estimate that more than 95% of the BAN-50/BA-27 and BAN-50/BC-05 signals directly measured in plasma were due to Aβ1–40 and Aβ1–42(43), respectively.

Plasma Aβ in subjects with familial or sporadic AD

In three separate studies, we performed blinded analyses of plasma Aβ in subjects with FAD-linked mutations or sporadic AD. These three studies assessed: (1) 12 carriers (seven presymptomatic and five symptomatic) and 31 noncarriers in the Swedish $APP_{KM670N,M671L}$ family; (2) 9 subjects that carried one of four different *PS1* mutations (one presymptomatic and eight symptomatic), 3 symptomatic subjects with the Volga German $PS2_{N141I}$ mutation and 1 symptomatic subject with the APP_{V717I} mutation, and 14 control subjects; and (3) 71 elderly patients with sporadic

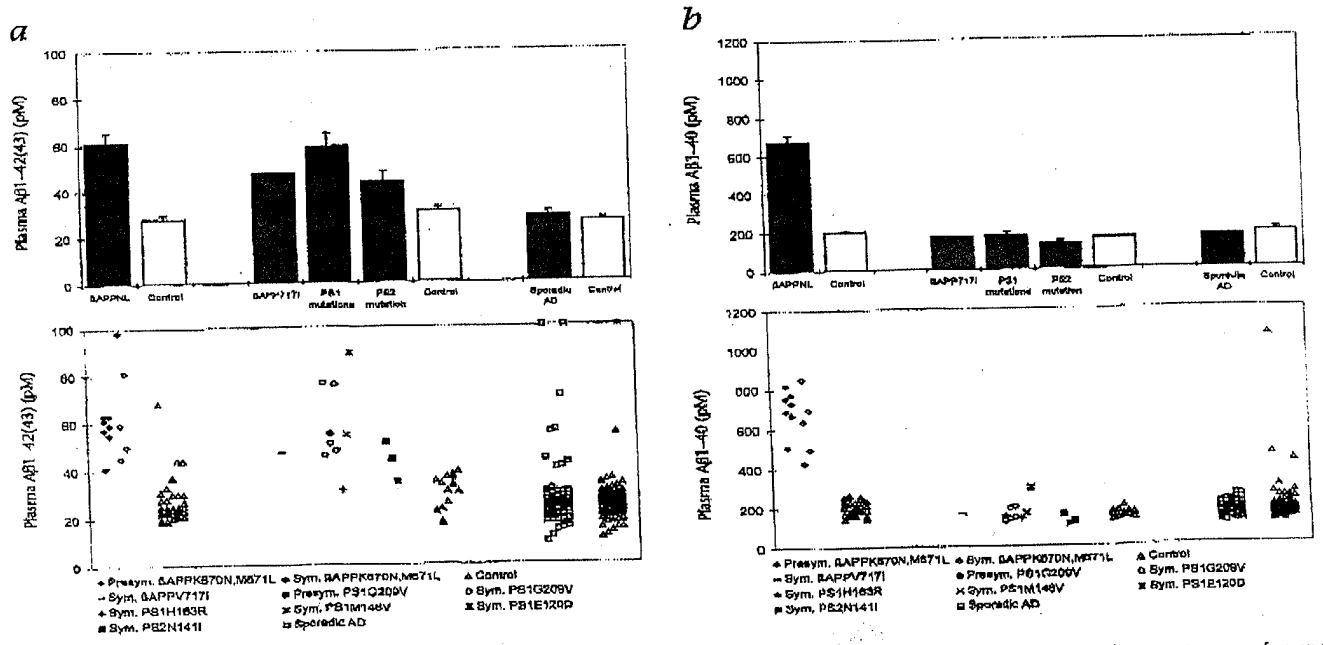

Fig. 2 Plasma Aβ1–42(43) (*a*) and Aβ1–40 (*b*) in subjects with FAD-linked mutations or sporadic AD. *upper panels*, Mean ± s.e.m. for each group; *lower panels*, values for the individual subjects in each group. Symptomatic and presymptomatic carriers of FAD-linked mutations and at-risk noncarriers were identified conventionally by PCR using appropriate primers.

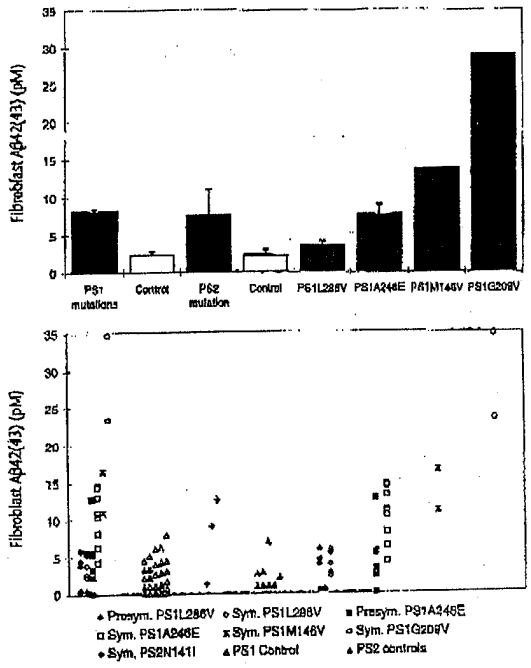

Fig. 3 Aβ42(43) in medium conditioned by fibroblasts from subjects with FAD-linked *PS1/2* mutations. We analyzed 26 fibroblast lines with *PS1* mutations and 30 control lines in a series of six experiments, and 3 lines with $PS2_{N141I}$ mutations and 8 additional control lines in a second series of four experiments. Each line was analyzed in 2–4 experiments; values plotted show the means for all measurements on day 5. *upper panel*, Mean ± s.e.m. for each group; *lower panel*, values for the individual subjects in each group. Carriers of FAD-linked mutations and at-risk noncarriers were identified conventionally by PCR using appropriate primers.

AD and 75 controls matched for age, sex and ethnicity. The statistical analysis of these plasma studies and the fibroblast studies that are reported below was performed using the nonparametric rank sum test, a conservative test that makes no assumptions about sample distribution. Because we specifically tested the hypothesis that the *PS1/2* mutations increase Aβ1–40 and/or Aβ1–42(43), the *P* values reported are for a one-tailed test. These values are one-half of those for the more conservative two-tailed test.

Plasma Aβ1–42(43) concentrations in the 31 controls of the first study ($APP_{K670N,M671L}$) (28 ± 2 pM), the 14 controls of the second (*PS1/2*) mutation study (30 ± 2 pM) and the 75 controls of the third (sporadic AD) study (27 ± 3 pM) were essentially identical (Fig. 2*a*). The mean concentration of plasma Aβ1–42(43) was highly significantly increased (Fig. 2*a*) both in the group of 8 symptomatic subjects with *PS1* mutations ($PS1_{G209V}$, $PS1_{M146V}$, $PS1_{I143R}$ or $PS1_{E280Q}$) (59 ± 7 pM, $P < 0.0001$) and in the 7 presymptomatic (57 ± 3 pM, $P < 0.0001$) and 5 symptomatic (67 ± 10 pM, $P = 0.0002$) subjects with $APP_{K670N,M671L}$ mutations, compared with the 45 age-matched controls. A similar increase in Aβ1–42(43) was observed in the single presymptomatic subject with a $PS1_{G209V}$ mutation (55 pM) and the single symptomatic subject with the APP_{V717I} mutation (47 pM). In the three symptomatic subjects with $PS2_{N141I}$ mutations (Fig. 2*a*), mean plasma Aβ1–42(43) was also significantly ($P = 0.009$) increased (43 ± 5 pM). Plasma Aβ1–40 in the 12 carriers with $APP_{K670N,M671L}$ mutations showed a marked, highly significant ($P < 0.0001$) increase that was essentially identical in the seven presymptomatic ($P < 0.0001$) and five symptomatic ($P = 0.0002$) carriers making up that group (Fig. 2*b*). Mean plasma Aβ1–40 did not, however, increase in sporadic AD or in subjects with *PS1/2* or APP_{V717I} mutations (Fig. 2*b*).

Thus, the mean concentration of Aβ1–42(43) was consistently increased in the plasma of subjects with each type of mutated gene known to cause early-onset familial AD, and this increase was unequivocally significant ($P < 0.0001$) when all 25 subjects with FAD-linked mutations were compared with all 45 age-matched controls analyzed in the first two studies. In the 12 subjects with *PS1* or *2* mutations, there was an unequivocal ($P < 0.0001$) selective increase in the mean concentration of Aβ1–42(43). As expected from previous studies[2,6–10], there was an increase in both Aβ1–40 and Aβ1–42(43) in subjects with the $APP_{K670N,M671L}$ mutation, but a selective increase in Aβ1–42(43) in the subject with an APP_{V717I} mutation.

In the third study, the mean concentration of Aβ1–42(43) was not significantly increased in the 71 subjects with late-onset

Table 1 Aβ from FAD (*PS1* mutant) and control fibroblasts, analyzed by experiment

Days of Conditioning	n	2	5	2	3	4	5	4	5
		βAPP synthesis		Aβ1-40 (pM)				Aβ1-42 (pM)	
Experiment 1									
Mean FAD1 ($S182A244G$)	7	1.58 ± 0.31	1.74 ± 0.31	3.6 ± 2.3	20.0 ± 8.0	30.0 ± 10.9	33.8 ± 11.1	17.2 ± 1.7**	12.5 ± 2.6^
Mean FAD2 ($S182L286V$)	4	1.74 ± 0.38	1.86 ± 0.53	13.2 ± 4.4*	22.0 ± 9.5*	26.7 ± 6.7^	44.0 ± 10.2*	3.2 ± 2.2	4.4 ± 3.0
Mean All FAD	11	1.64 ± 0.23	1.78 ± 0.26	7.2 ± 2.5	21.0 ± 5.9*	28.8 ± 7.1*	37.5 ± 7.8*	12.1 ± 2.9^	9.5 ± 2.2^
Mean Control	7	1.56 ± 0.26	1.36 ± 0.21	2.2 ± 1.5	3.6 ± 2.6	6.1 ± 3.9	14.6 ± 4.9	1.6 ± 1.1	1.4 ± 1.4
	n	βAPP synthesis		Aβ1-40/βAPP synthesis				Aβ1-42/βAPP syn.	
Experiment 1									
Mean FAD1 ($S182A244G$)	7	1.58 ± 0.31	1.74 ± 0.31	1.8 ± 1.1	10.0 ± 3.9	14.4 ± 5.2	18.4 ± 4.2	13.5 ± 3.3*	8.4 ± 1.3**
Mean FAD2 ($S182L286V$)	4	1.74 ± 0.38	1.86 ± 0.53	7.1 ± 3.0*	13.4 ± 7.8	18.2 ± 5.9*	29.0 ± 9.5*	1.7 ± 1.0	2.4 ± 1.4
Mean All FAD	11	1.64 ± 0.23	1.78 ± 0.26	3.7 ± 1.6	11.2 ± 3.5*	15.8 ± 3.8*	22.3 ± 4.4*	9.2 ± 2.7^	6.2 ± 1.3^
Mean Control	7	1.56 ± 0.26	1.36 ± 0.21	1.0 ± 0.7	2.0 ± 1.3	6.1 ± 3.9	9.1 ± 2.9	0.9 ± 0.60	0.7 ± 0.7
Experiment 2									
Mean Family FAD ($S182G209V$)	2	1.50 ± 0.70	1.24 ± 0.50	14.4 ± 2.5	32.6 ± 8.0	60.0 ± 11.8	48.9 ± 15.1	22.7 ± 5.3	NM
Mean Control	2	1.68 ± 0.01	1.99 ± 0.21	7.0 ± 3.2	11.3 ± 2.4	41.4 ± 7.3	20.1 ± 4.6	2.0 ± 2.0	NM
Experiment 3									
Mean FAD1 ($S182A244G$)	4	NM	1.17 ± 0.37	NM	NM	NM	51.8 ± 17.3*	NM	10.9 ± 2.6**
Mean FAD2 ($S182L286V$)	5	NM	1.95 ± 0.11	NM	NM	NM	37.7 ± 0.3*	NM	5.5 ± 1.4**
Mean Family FAD ($S182G209V$)	2	NM	1.20 ± 0.36	NM	NM	NM	96.0 ± 25.1	NM	35.3 ± 5.9**
Mean All FAD	13	NM	1.47 ± 0.20	NM	NM	NM	52.9 ± 10.1^	NM	12.9 ± 3.3**
Mean Control	11	NM	1.19 ± 0.27	NM	NM	NM	23.1 ± 4.1	NM	1.0 ± 0.4
Experiment 4									
Mean Swed/Finn FAD ($S182M146V$)	2	1.45 ± 0.26	1.16 ± 0.18	42.4 ± 0.2	NM	NM	105.0 ± 24.1	NM	21.2 ± 4.8
Mean Control	5	1.69 ± 0.14	1.80 ± 0.26	43.9 ± 1.6	NM	NM	80.6 ± 9.0	NM	8.4 ± 1.9
Experiment 5									
Mean Swed/Finn FAD ($S182M146V$)	2	NM	1.35 ± 0.26	37.2 ± 4.7	27.4 ± 5.5	43.7 ± 3.0	57.3 ± 7.8	5.1 ± 2.0	6.2 ± 0.8
Mean Control	5	NM	1.73 ± 0.16	27.6 ± 3.0	23.6 ± 4.2	40.3 ± 6.1	38.2 ± 8.6	2.9 ± 0.4	2.2 ± 0.4
Experiment 6									
Mean presym. FAD1 ($S182A244G$)	6	0.92 ± 0.39	0.72 ± 0.44	40.3 ± 13.4^	30.6 ± 8.5^	30.1 ± 7.7	49.9 ± 13.9*	3.1 ± 1.1	6.4 ± 4.0
Mean presym. FAD2 ($S182L286V$)	5	2.20 ± 0.32	2.22 ± 0.59	19.2 ± 3.6	9.4 ± 3.1	11.8 ± 4.5	13.4 ± 6.6	2.3 ± 0.8	3.7 ± 1.4
Mean all presym. FAD	11	1.56 ± 0.32	1.47 ± 0.43	29.3 ± 8.3	20.1 ± 5.6	20.9 ± 6.2	31.6 ± 9.4	2.7 ± 0.7	5.0 ± 2.1
Mean Control	13	1.66 ± 0.22	1.73 ± 0.23	12.3 ± 2.2	12.0 ± 2.8	16.5 ± 3.8	20.5 ± 5.5	1.9 ± 0.4	3.0 ± 0.7

*$P < 0.05$; ^$P < 0.01$; **$P < 0.001$ by Student's unpaired *t*-test.

Table 2 Aβ from FAD fibroblasts (means of all experiments, day 5)

Cell line	Family	Status	Aβ/βAPP synthesis (pM/norm. pixels X 10-5) Aβ1-40	Aβ42(43)
AG07637 A	FAD1 (*PS1* A246E)	AD	25.2	10.4
AG07613A	FAD1 (*PS1* A246E)	AD	13.5	14.5
AG04159A	FAD1 (*PS1* A246E)	AD	62.4	13.1
AG08844C	FAD1 (*PS1* A246E)	AD	38.6	11.0
AG08840B	FAD1 (*PS1* A246E)	AD	15.3	4.3
AG08848B	FAD1 (*PS1* A246E)	AD	37.6	8.2
AG08170A	FAD1 (*PS1* A246E)	AD	12.7	6.3
AG07671	FAD1 (*PS1* A246E)	Presymp	50.2	3.3
AG08168	FAD1 (*PS1* A246E)	Presymp	44.1	2.3
AG08178	FAD1 (*PS1* A246E)	Presymp	40.7	5.5
AG07617	FAD1 (*PS1* A246E)	Presymp	64.2	12.7
AG07689	FAD1 (*PS1* A246E)	Presymp	0.7	0.0
Mean ± SE FAD1 lines (12)			35.4 ± 6.8*	7.6 ± 1.4**
AG08527A	FAD2 (*PS1* L286V)	AD	36.4	5.8
AG08541A	FAD2 (*PS1* L286V)	AD	19.6	3.9
AG08555	FAD2 (*PS1* L286V)	AD	25.3	2.7
AG08563A	FAD2 (*PS1* L286V)	AD	57.1	5.4
AG08587	FAD2 (*PS1* L286V)	AD	26.8	2.3
AG08187	FAD2 (*PS1* L286V)	Presymp	20.5	4.5
AG08545	FAD2 (*PS1* L286V)	Presymp	14.2	3.9
AG09171	FAD2 (*PS1* L286V)	Presymp	26.5	5.9
AG08507	FAD2 (*PS1* L286V)	Presymp	0.5	0.4
AG09177	FAD2 (*PS1* L286V)	Presymp	1.4	0.4
Mean ± SE FAD2 lines (10)			22.6 ± 5.2	3.5 ± 0.6
LB01	L family (*PS1* Q209V)	AD	50.9	23.4
LB02	L family (*PS1* Q209V)	AD	91.0	34.7
Mean L family lines (2)			70.9*	29.0**
KH	Sw/Finn (*PS1* M146V)	AD	90.3	16.5
UBH	Sw/Finn (*PS1* M146V)	AD	71.8	11.0
Mean Swed/Finn lines (2)			81.0*	13.8**
Mean ± SE all *PS1* lines (26)			36.8 ± 5.2*	8.2 ± 1.5†
VG1	VG (*PS2*N141I)	AD	52.6	12.6
VG2	VG (*PS2*N141I)	AD	43.2	9.0
VG3	VG (*PS2*N141I)	AD	5.9	1.2
Mean ± SE *PS2*N141I lines (3)			22.6 ± 5.2*	7.6 ± 3.4*

†$P < 0.0001$; **$P < 0.01$; *$P < 0.05$ by one-tailed rank sum test compared with 38 control lines in Table 3.

Table 3 Aβ from control fibroblasts (means of all experiments, day 5)

Cell line	Family	Aβ/βAPP synthesis (pM/norm. pixels X 10-5) Aβ1-40	Aβ42(43)
AG07619A	FAD1	9.1	0.7
AG07583A	FAD1	0.0	0.0
AG07623A	FAD1	15.9	0.0
AG08701	FAD1	19.2	0.8
AG08178A	FAD1	10.1	0.0
AG07671	FAD1	31.9	1.0
AG08703	FAD1	30.1	2.9
AG07376	FAD1	2.8	0.0
AG07579A	FAD1	4.4	0.7
AG07597	FAD1	3.2	0.3
AG08509	FAD2	2.9	0.0
AG08517	FAD2	16.8	0.0
AG08529	FAD2	33.6	2.1
AG08539	FAD2	17.5	0.0
AG08543	FAD2	18.3	3.4
AG09181	FAD2	29.0	4.3
AG08535	FAD2	51.4	4.5
AG08537	FAD2	29.1	3.2
AG09175	FAD2	24.7	6.0
AG08515	FAD2	1.8	1.0
AG08561	FAD2	30.0	4.0
AG08567	FAD2	1.2	1.2
AG08533	FAD2	1.0	2.2
JY34	Swed/Finn	72.7	4.5
JY66	Swed/Finn	48.4	6.2
SY	Swed/Finn	49.2	3.0
AH56	Swed/Finn	63.5	7.9
SN56	Swed/Finn	53.1	5.1
LB44	L family	38.8	1.6
LB45	Lfamily	27.3	2.6
VGC1	VG	5.7	1.0
VGC2	VG	24.7	2.8
VGC3	VG	7.6	1.0
VGC4	VG	22.5	2.2
VGC5	VG	8.4	0.6
VGC6	VG	10.3	0.8
VGC7	VG	41.2	6.6
VGC8	VG	12.6	2.6
Mean ± SE control lines (38)		23.2 ± 3.1	2.3 ± 0.3

sporadic AD (29 ± 2 pM) (Fig. 2*b*) compared with the 75 age-matched control subjects (27 ± 3 pM). This observation and our finding that Aβ1–42(43) was significantly increased in all eight presymptomatic gene carriers (seven with $APP_{K670N,M671L}$ mutations and one with a $PS1_{L286V}$ mutation) indicate that the increased Aβ1–42(43) observed in subjects with FAD-linked *APP* and *PS1/2* mutations occurs as a direct consequence of the mutations and not as an indirect manifestation of the AD state or of altered nutrition or drug intake that might be associated with the AD state.

Aβ in medium conditioned by fibroblasts from FAD subjects

To obtain further and independent evidence that the FAD-linked *PS1/2* mutations increase Aβ42(43), we quantified the Aβ secreted by primary skin fibroblasts, since these cells are known to express the *PS1* (L. Levesque *et al.*, manuscript submitted) and *PS2* (ref. 17) genes. Conditioned medium was analyzed on days 2–5 *in vitro* for Aβ1–40 (BAN-50/BA-27 ELISA) and on days 4 and 5 for Aβ ending at Aβ42(43) (BC-05/4G8 or BAN-50/BC-05 ELISA)[2,29]. As a denominator, βAPP synthesized during 20-minute labeling with [^{35}S]methionine was quantified in the lysate of each fibroblast line. The Aβ1–40 and Aβ ending at Aβ42(43) [Aβ42(43)] secreted by each line were normalized for that line's βAPP synthesis to assess Aβ1–40 and Aβ42(43) accumulation per molecule of βAPP synthesized.

Control fibroblasts and fibroblasts with FAD-linked *PS1* mutations showed no significant difference in βAPP synthesis (Table 1). Our initial analysis of 5-day conditioned medium from 11 FAD (seven FAD1, four FAD2) and 7 control lines matched for age and passage number, showed significant increases in Aβ1–40 ($P < 0.05$) and Aβ42(43) ($P < 0.01$) both before and after normalization for βAPP synthesis (Table 1, Experiment 1). We pursued these observations by performing five more experiments in which we compared a total of 30 control lines and 26 lines with FAD-linked *PS1* mutations. In the six experiments, we made 15 comparative measurements of (Aβ42(43)/βAPP synthesis) in control fibroblasts versus groups of fibroblasts with four different *PS1* mutations (Table 1), and in each comparison, Aβ42(43)/βAPP synthesis in the *PS1* mutation lines always exceeded that in the control lines (Table 1).

In a second set of four experiments, we compared three fibroblast lines from subjects with $PS2_{N141I}$ mutations with eight other control lines. In these four experiments, we made six comparative measurements of (Aβ42(43)/βAPP synthesis) in the group of three lines with $PS2_{N141I}$ mutations and the group of eight control lines, and in each comparison, Aβ42(43)/βAPP synthesis in the $PS2_{N141I}$ mutation lines again exceeded that in the controls. The values obtained (means ± s.e.m.) for (Aβ42(43)/βAPP synthesis) and (Aβ1–40/βAPP synthesis) in the 29 lines with *PS1* or *PS2* mutations and the 38 control lines are listed in Tables 2 and 3, respectively.

The amount of Aβ secreted by fibroblasts varied considerably from line to line (Fig. 3). Nonetheless, in 5-day conditioned

medium, there was a highly significant ($P < 0.0001$) overall increase in mean Aβ42(43)/βAPP synthesis in the 26 lines with *PS1* mutations (8.2 ± 1.5 pM) as compared with the 38 control lines (2.3 ± 0.3 pM) (Fig. 3, Tables 2 and 3). When we compared mean Aβ42(43)/βAPP synthesis in the 38 controls to specific groups of mutant lines, we found (1) significant increases in the two $PS1_{C280V}$ and two $PS1_{M146V}$ lines from symptomatic subjects ($P = 0.009$ for each mutation); (2) a significant ($P = 0.0002$) increase in the twelve $PS1_{A246E}$ lines (seven symptomatic and five presymptomatic); and (3) a significant ($P = 0.03$) increase in the three symptomatic $PS2_{N141I}$ lines. The ten lines with $PS1_{A246V}$ mutations (five symptomatic and five presymptomatic) also had elevated mean Aβ42(43)/βAPP synthesis, but this increase was smaller than that observed for the other mutations and did not reach statistical significance ($P = 0.052$).

We also observed a significant ($P = 0.02$) overall increase in (Aβ1–40/βAPP synthesis) in the 26 lines with PS1 mutations (36.8 ± 5.2) as compared with the 38 controls (23.2 ± 3.1). Significant increases in Aβ1–40/βAPP synthesis were observed in the lines with $PS1_{A246E}$ ($P = 0.05$), $PS1_{C280V}$ ($P = 0.02$), and $PS1_{M146V}$ ($P = 0.01$) mutations but there was no increase in the lines with a $PS1_{C280V}$ mutation and the increase in lines with a $PS2_{N141I}$ mutation was not significant ($P = 0.17$) (Tables 2 and 3). Thus Aβ1–40/βAPP synthesis appeared to increase in fibroblasts with some FAD-linked *PS1/2* mutations, but the effect was not nearly as pronounced or definite as that observed for Aβ42(43)/βAPP synthesis.

Discussion

Our study strongly suggests that a fundamental, generalized effect of the FAD-linked *APP*, *PS1* and *PS2* mutations is to increase the extracellular concentration of Aβ42(43). The plasma data are particularly important because they establish that these mutations increase extracellular Aβ42(43) *in vivo*. This effect is likely to be directly related to the pathogenesis of AD, because Aβ42(43) is deposited early[1] and selectively[2–4] in the senile plaques that are an invariant feature of all forms of AD. Thus our results suggest that the FAD-linked mutations may all cause AD by increasing the extracellular concentration of Aβ42(43), thereby fostering Aβ deposition, and they support the hypothesis that cerebral Aβ deposition is an essential early event in the pathogenesis of AD. Although this makes reduction of Aβ concentration and prevention of Aβ deposition attractive as therapeutic targets in AD, it does not mean that pathologic changes that may result from increased Aβ concentration and/or Aβ deposition (such as paired helical filament formation) are poor therapeutic targets, because the utility of preventing any pathologic change in AD depends on its importance in the development of dementia rather than on its relative position in the pathologic cascade that produces dementia.

It is possible that it is not Aβ deposition *per se* that triggers AD pathogenesis but another change caused by increased extracellular Aβ42(43), such as the formation of soluble complexes containing Aβ42(43) that are toxic. Toxic, soluble complexes of this sort could, in principle, foster incidental Aβ deposition that is not critically important for AD pathogenesis, even though Aβ deposition and the changes associated with it would invariably occur during the pathogenic process. It is also possible that the FAD-linked mutations all initiate as-yet-unidentified molecular changes that lead to both a cascade of increased Aβ42(43) concentration, Aβ deposition, and perhaps even neuritic plaque formation that is unrelated to the development of dementia, and a separate pathologic cascade, possibly involving paired helical filament formation, that does lead to dementia. Given the burgeoning evidence that Aβ deposition or something closely linked to Aβ deposition is toxic *in vitro* and *in vivo* and the lack of evidence that the FAD-linked mutations produce changes unrelated to increased Aβ42(43) that are involved in AD pathogenesis, we think that it is highly unlikely that the increased extracellular concentration of Aβ42(43) produced by the FAD-linked mutations is an epiphenomenon. Importantly, plasma Aβ42(43) was increased in all of the presymptomatic carriers that we examined, and it was not increased in the vast majority of symptomatic sporadic subjects. Thus elevated Aβ42(43) is not a secondary phenomenon of the AD state.

The mechanism underlying the increase in Aβ42(43) caused by *PS1/2* mutations remains unclear. It has been suggested that the presenilins may be involved in the intracellular trafficking of membranous vesicles[15]. Thus, the presenilin missense mutations may alter membrane protein trafficking in a way that subtly enhances the exposure of βAPP to the γ secretase that cleaves at Aβ42, thereby increasing Aβ42(43) generation.

It is, in our view, unlikely that cerebral Aβ42(43) deposition is a direct result of the increased plasma Aβ42(43) we report in subjects with FAD-linked *APP* and *PS1/2* mutations. Rather, Aβ42(43) deposition is presumably due to an increase in its extracellular concentration in the brain that occurs as part of a generalized effect of these mutations in neural and nonneural cells, all of which are known to express βAPP and to secrete Aβ constitutively. It was reported recently that Aβ42 declines in cerebrospinal fluid (CSF) samples from some patients with sporadic AD (ref. 31). To explain this finding, it was suggested that preferential deposition of Aβ42(43) as insoluble deposits in AD brain may lead to reduced CSF levels of the soluble peptide, as occurs in another inherited CNS amyloid disease, cystatin C amyloidosis in Icelandic families, in which the level of the amyloid-forming protein is also reduced in the CSF of affected individuals undergoing progressive amyloid deposition[32]. Once cerebral Aβ deposition is under way, the data of Motter *et al.*[31] suggest that the concentration of CSF Aβ42(43) will decline, often to levels that are lower than normal. Because Aβ42(43) deposition apparently occurs long before symptoms are evident, it will be important to examine Aβ42(43) CSF levels both in young carriers (in whom Aβ deposition may be minimal) and in symptomatic carriers to establish that CSF Aβ42(43) is elevated initially in subjects with *PS1/2* mutations, as suggested by our data, but declines when cerebral Aβ deposition accelerates as suggested by Motter *et al.*[31].

Increasing extracellular Aβ42(43) concentration is only one of several mechanisms that could foster the cerebral Aβ deposition that invariably occurs in AD. Our results suggest that the mutations that cause early-onset FAD may all act through this mechanism, but our data also show that the Aβ42(43) deposition that occurs in most sporadic AD patients is not caused by a generalized increase in extracellular Aβ42(43) concentration that is evident in plasma. In sporadic AD, cerebral deposition of Aβ42(43) must be caused by other factors, such as a local increase in the secretion of Aβ42(43), alterations in Aβ binding proteins (for example, ApoE, ref. 33–36) that increase the rate of deposition, or an impairment of the cerebral mechanisms that normally remove soluble or deposited Aβ.

Although most of the sporadic AD patients that we examined clearly did not have increased plasma Aβ42(43), inspection of the data from 71 sporadic AD patients and 75 controls (Fig. 2) shows that in 11 of the 146 subjects examined, Aβ1–42(43) was

elevated (Fig. 2*b*) into the range observed in subjects with the FAD-linked *APP*, *PS1* and *PS2* mutations. In this group of 11, the frequency of sporadic AD was substantially and significantly ($P < 0.03$) increased. Nine of these 11 had sporadic AD and the two unaffected individuals were younger subjects still at risk for AD. Five of the 11 were over the age of 80 and each had sporadic AD. Remarkably, two of the nine subjects with elevated plasma Aβ1–42(43) showed this elevation before the onset of clinically apparent disease — they were in the control group initially and subsequently developed AD. Thus it is tempting to speculate that an elevated concentration of Aβ1–42(43) that is detectable in plasma may play a part in 10–20% of sporadic AD cases and that this elevation may be present before symptoms develop. Further studies are needed to determine whether individuals who have elevated plasma Aβ1–42(43) are, in fact, at greater risk of developing AD and, if so, whether there is a genetic basis for their AD.

Methods

Analysis of plasma Aβ1–40 and Aβ1–42(43). Symptomatic and presymptomatic carriers of FAD-linked mutations and at-risk noncarriers were identified conventionally by PCR using appropriate primers. For the study of sporadic AD, probable AD patients who were part of an Alzheimer's Disease Patient Registry[37] and corresponding control subjects of similar age and sex who were also enrolled in a larger, epidemiological AD case-control study[38] were evaluated by consecutive identification number. The clinical diagnosis of AD was based on National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association criteria[39]. Blood was drawn into tubes containing EDTA. As rapidly as possible, cellular material was pelleted by centrifugation. Plasma was then frozen in 1-ml aliquots and stored at –70° C. To assay Aβ1–40 and Aβ1–42(43), plasma (300 µl) was mixed with 525 µl of EC buffer[29] and 75 µl of CNBr-activated Sepharose beads (Pharmacia) covalently cross-linked to a nonspecific IgG1k monoclonal antibody. This mixture was rocked at 4 °C for 2 h and the beads removed by centrifugation. This preabsorption with nonspecific IgG1k reduces signal associated with nonspecific proteins and is particularly helpful in the BAN-50/BC-05 assay. Ninety-six-well microtiter plates that had been coated with BAN-50 (ref. 29) were washed twice with PBS (100 mM phosphate, 150 mM NaCl, pH 7.4), and 50 µl of EC buffer was added to each well to prevent drying. The preabsorbed supernatant (100 µl) was then added in duplicate to the microtiter wells and BA-27-HRP or BC-05-HRP were used to detect Aβ1–40 or Aβ1–42(43), respectively, as described[29]. In addition to experimental plasma samples, each plate contained known concentrations of synthetic Aβ1–40 or Aβ1–42 in EC buffer (used to construct a standard curve for determining the concentration of Aβ in each of the plasma samples) and known plasma samples from young volunteers that were used as standards to normalize the values obtained on each plate.

Specificity of plasma Aβ1–40 and Aβ1–42(43) assays. Plasma (50 ml) was applied to a BAN-50 column consisting of 0.75 mg of the antibody immobilized to 0.1 g of Tresyl Toyopearl resin. Adsorbed materials were eluted with 1 ml of 60% CH_3CN containing 0.2% trifluoroacetic acid (TFA), and the eluate was lyophilized and further fractionated by reversed-phase HPLC on a Vydac C4 column (4.6 × 250 mm). In the fractionation, CH_3CN concentration (containing 0.1% TFA) was kept at 23.75% for the first 5 min and then linearly increased from 23.75 to 32.75% over 60 min at a flow rate of 0.5 ml/min. Aliquots from fractions were analyzed by BAN-50/BA-27 or BAN-50/BC-05 ELISA (ref. 29). When a BAN-50/BC-05 sandwich ELISA is used for analysis, synthetic Aβ1–43 is detected with less sensitivity than Aβ1–42 by a factor of 10. Since some of the plasma Aβ detected by BAN-50/BC-05 ELISA may be Aβ1–43, this Aβ is referred to as Aβ1–42(43). Synthetic Aβ1–40 and Aβ1–42 run immediately before the eluate from the BAN-50 column, eluted, respectively, in fraction 79, and as a broad peak in fractions 85–90. Thus, in plasma, as in medium conditioned by transfected cells[29], the BAN-50/BA-27 and BAN-50/BC-05 ELISAs recognize Aβs that coelute with synthetic Aβ1–40 and Aβ1–42, respectively. Recovery of Aβ applied to the BAN-50 column was ~40% for both Aβ1–40 and Aβ1–42. Recovery of Aβ1–40 and Aβ1–42 from the C4 column was 66% and 28%, respectively. Assuming similar recovery of the Aβ1–40 and Aβ1–42(43) in plasma, we found that more than 95% of the BAN-50/BA-27 and BAN-50/BC-05 signals directly measured in plasma were due to Aβ1–40 and Aβ1–42(43), respectively.

Analysis of Aβ1–40 and Aβ42(43) secreted by fibroblasts. Fibroblasts were cultured in minimum essential medium (MEM) containing 10% FCS, penicillin, streptomycin, glutamine and 10 mM HEPES, pH 7.4. Sister cultures for analyzing βAPP synthesis or the Aβ in medium conditioned 2–5 days were processed in parallel, plating initially at 80% confluence. Conditioned medium, stored frozen at –70 °C, was thawed and analyzed for Aβ1–42(43) (BAN-50/BC-05 ELISA)[29] or with a BC-05/4G8(anti-Aβ17-24) ELISA that measures both Aβ1–42(43) and N-terminally truncated Aβs ending at Aβ42(43) (ref. 2) (for example, Aβ17–42(43)). It is preferable to use a BC-05/4G8 assay rather than a BAN-50/BC-05 assay when measuring low-level Aβ ending at Aβ42(43) because 4G8-HRP produces less background signal than BC-05-HRP. Thus, we employed the BC-05/4G8 assay in our initial studies of fibroblast with *PS1* mutations. To evaluate Aβ1–42(43) in the experiments on three $PS2_{N141I}$ lines versus eight control lines, we prepared multiple batches of BC-05-HRP. By selecting a batch with particularly low background, we were able to use a BAN-50/BC-05 assay to analyze the Aβ1–42(43) secreted by these fibroblasts. These measurements showed that the concentrations of Aβ1–42(43) (BAN-50/BC-05 assay) and AβX–42(43) (BC-05/4G8 assay) were essentially identical, indicating that in fibroblast medium both assays measure Aβ1–42(43). This result is concordant with previously published data[26] showing that human skin fibroblasts differ from many other cells in that fibroblasts overwhelmingly produce full-length 4-kDa Aβ and very little P3. To assess βAPP synthesis, cells were pulse-labeled for 20 min with ICN TRANS^{35}S-LABEL and the newly synthesized radiolabeled βAPP was immunoprecipitated and quantified by phosphorimaging[27]. Values for (Aβ42(43)/βAPP synthesis) were calculated by dividing the concentration of Aβ42(43) for each cell line by the βAPP synthesis for that line.

Acknowledgments

We thank D. Glass and D. Yager for excellent technical assistance, Karin Axelman, Charlotte Forsell and Lena Lilius for valuable help with the sample collection, the laboratory members who donated blood, Hans Basun and Dale Schenk for valuable discussions, and Bengt Winblad for generous support. This work was supported by the Alzheimer's Disease and Related Disorders Association (S.Y.), the American Health Assistance Foundation (J.H., S.Y.), the Axelsson-Johnsson, Osterman, Soderstrom-Konig, Magnus Bergvall, and Gamla Tjanarinnor foundations (L.L.), the Bank of Sweden Tercentenary Foundation (L.L.), the Swedish Medical Research Council (L.L.), and grants from the US National Institutes of Health (T.D.B., J.H., E.P., G.S., R.T., D.S. and S.Y.).

RECEIVED 28 MARCH; ACCEPTED 3 JUNE 1996

1. Iwatsubo, T., Mann, D.M., Odaka, A., Suzuki, N. & Ihara, Y. Amyloid beta protein (Aβ) deposition: Aβ42(43) precedes Aβ40 in Down syndrome. *Ann. Neurol.* 37, 294–299 (1995).
2. Gravina, S.A. *et al.* Amyloid beta protein (Aβ) in Alzheimer's disease brain. Biochemical and immunocytochemical analysis with antibodies specific for forms ending at Aβ40 or Aβ42(43). *J. Biol. Chem.* 270, 7013–7016 (1995).
3. Roher, A. *et al.* Structural alterations in the peptide backbone of β-amyloid core protein may account for its deposition and stability in Alzheimer's disease. *J. Biol. Chem.* 268, 3072–3083 (1993).
4. Miller, D.L. *et al.* Peptide compositions of the cerebrovascular and senile plaque core amyloid deposits of Alzheimer's disease. *Arch. Biochem.* 301, 41–52 (1993).
5. Seubert, P. *et al.* Isolation and quantification of soluble Alzheimer's beta-peptide from biological fluids. *Nature* 359, 325–327 (1992).
6. Shoji, M. *et al.* Production of the Alzheimer amyloid beta protein by normal proteolytic processing. *Science* 258, 126–129 (1992).
7. Haass, C. *et al.* Amyloid beta-peptide is produced by cultured cells during normal metabolism. *Nature* 359, 322–325 (1992).
8. Busciglio, J. Gabuzda, D.H., Matsudaira, P. & Yankner, B.A. Generation of beta-amyloid in the secretory pathway in neuronal and nonneuronal cells. *Proc. Natl. Acad. Sci. USA* 90, 2092–2096 (1993).
9. Dovey, H.F., Suomesaari-Chrysler, S., Lieberburg, I., Sinha, S. & Kiem, P.S. Cells with a familial Alzheimer's disease mutation produce authentic β-peptide. *NeuroReport* 4, 1039–1042 (1993).
10. Vigo-Pelfrey, C., Lee, D., Keim, P., Lieberburg, I. & Schenk, D. B. Characterization of β amyloid peptide from human cerebrospinal fluid. *J. Neurochem.* 61, 19965–19968 (1993).
11. Hilbich, C., Kisters-Wolke, B., Reed, J., Masters, C.L. & Beyreuther, K. Aggregation and secondary structure of synthetic amyloid βA4 peptides of Alzheimer's disease. *J. Mol. Biol.* 218, 149–163 (1991).
12. Burdick, D. *et al.* Assembly and aggregation properties of synthetic Alzheimer's A4/β amyloid peptide analogs. *J. Biol. Chem.* 267, 546–554 (1992).
13. Jarrett, J.T. & Lansbury, P.T. Jr. Seeding "one-dimensional crystallization" of amyloid: A pathogenic mechanism in Alzheimer's disease and scrapie? *Cell* 73, 1055–1058 (1993).
14. Jarrett, J.T., Berger, E.P. & Lansbury, P.T. Jr. The carboxy terminus of β amyloid protein is critical for the seeding of amyloid formation: Implications for pathogenesis of Alzheimer's disease. *Biochemistry* 32, 4693–4697 (1993).
15. Sherrington, R. *et al.* Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature* 375, 754–760 (1995).
16. Levy-Lahad, E. *et al.* Candidate gene for the chromosome 1 familial Alzheimer's disease locus. *Science* 269, 973–977 (1995).
17. Levy-Lahad, E. *et al.* A familial Alzheimer's disease locus on chromosome 1. *Science* 269, 970–973 (1995).
18. Rogaev, E. *et al.* Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. *Nature* 376, 775–778 (1995).
19. Goate, A. *et al.* Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* 349, 704–706 (1991).
20. Naruse, S. *et al.* Missense mutation Val–Ile in exon 17 of amyloid precursor protein gene in Japanese familial Alzheimer's disease. *Lancet* 337, 978 (1991).
21. Yoshioka, K., Miki, T., Katsuya, T., Ogihara, T. & Sakaki, Y. The 717Val–Ile substitution in amyloid precursor protein is associated with familial Alzheimer's disease regardless of ethnic groups. *Biochem. Biophys. Res. Commun.* 176, 1141 (1991).
22. Hardy, J. *et al.* Molecular classification of Alzheimer's disease. *Lancet* 337, 1342 (1991).
23. Murrell, J., Farlow, M., Ghetti, B. & Benson, M.D. A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. *Science* 254, 97–99 (1991).
24. Chartier-Harlin, M.-C. *et al.* Early-onset Alzheimer's disease caused by mutations at codon 717 of the β-amyloid precursor protein gene. *Nature* 353, 844–846 (1991).
25. Mullan, M. *et al.* A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of β-amyloid. *Nature Genet.* 1, 345–347 (1992).
26. Citron, M. *et al.* Excessive production of amyloid beta-protein by peripheral cells of symptomatic and presymptomatic patients carrying the Swedish familial Alzheimer disease mutation. *Proc. Natl. Acad. Sci. USA* 91, 11993–11997 (1994).
27. Cai, X.D., Golde, T.E. & Younkin, S.G. Release of excess amyloid beta protein from a mutant amyloid beta protein precursor. *Science* 259, 514–516 (1993).
28. Citron, M. *et al.* Mutation of the β-amyloid precursor protein in familial Alzheimer's disease increases β-protein production. *Nature* 360, 672–674 (1992).
29. Suzuki, N. *et al.* An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (β APP717) mutants. *Science* 264, 1336–1340 (1994).
30. Tamaoka, A. *et al.* APP717 missense mutation affects the ratio of amyloid beta protein species (Aβ1–42/43 and Aβ1–40) in familial Alzheimer's disease brain. *J. Biol. Chem.* 269, 32721–32724 (1994).
31. Motter, R. *et al.* Reduction of beta-amyloid peptide 42 in the cerebrospinal fluid of patients with Alzheimer's disease. *Ann. Neurol.* 38, 643–648 (1995).
32. Grubb, A. *et al.* Abnormal metabolism of gamma-trace alkaline microprotein. The basic defect in hereditary cerebral hemorrhage with amyloidosis. *N. Engl. J. Med.* 311, 1547–1549 (1984).
33. Ma, J., Yee, A., Brewer, H.B. Jr., Das, S. & Potter, H. Amyloid-associated proteins alpha 1-antichymotrypsin and apolipoprotein E promote assembly of Alzheimer beta-protein into filaments. *Nature* 372, 92–94 (1994).
34. Wisniewski, T., Castano, E.M., Golabek, A. Vogel, T. & Frangione B. Acceleration of Alzheimer's fibril formation by apolipoprotein E in vitro. *Am. J. Pathol.* 145, 1030–1035 (1994).
35. Castaño, E. *et al.* Fibrillogenesis in Alzheimer's disease of the amyloid beta peptides and apolipoprotein E. *Biochem. J.* 306, 599–604 (1995).
36. Evans, K.C., Berger, E.P., Cho, C.G., Weisgraber, K.H. & Lansbury, P.T. Jr. Apolipoprotein E is a kinetic but not a thermodynamic inhibitor of amyloid formation: Implications for the pathogenesis and treatment of Alzheimer's disease. *Proc. Natl. Acad. Sci. USA* 92, 763–767 (1995).
37. Larson, E.B. *et al.* University of Washington Alzheimer's Disease Patient Registry (ADPR): 1987–1988. *Aging* 2, 404–408 (1990).
38. Kukull, W.A. *et al.* Solvent exposure as a risk factor for Alzheimer's disease: A case-control study. *Am. J. Epidemiol.* 141, 1059–1071 (1995); (erratum) *Am. J. Epidemiol.* 142, 450 (1996).
39. McKhann, G. *et al.* Clinical diagnosis of Alzheimer's disease: Report of the NINCSDS–ADRDA Work Group under the auspices of the Department of Health and Human Services Task Force on Alzheimer's disease. *Neurology* 34, 939–944 (1984).